#21 Exhibit, attach

PATENT
Our Docket: P-PM 3474

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Seidman and Théorêt	Group Art Unit: 1623
Serial No.: 09/288,344	Examiner: L. Crane
Filed: April 8, 1999	
For: METHODS OF OPTIMIZING DRUG THERAPEUTIC EFFICACY FOR TREATMENT OF IMMUNE-MEDIATED GASTROINTESTINAL DISORDERS	

Commissioner for Patents
Washington, D.C. 20231

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Sir:

I, Stephan R. Targan, declare as follows:

1) I am currently a Professor of Distinction in the Department of Medicine, Division of Gastroenterology, University of California, Los Angeles, School of Medicine; Director, Cedars-Sinai Medical Center Inflammatory Bowel Disease Center; Director, Cedars-Sinai Medical Center, Division of Gastroenterology; and Feintech Family Chair in Inflammatory Bowel Disease.

2) I received a doctorate in medicine from Johns Hopkins University. My internship and residency were performed at Harbor-UCLA Medical Center from 1971 to 1974.

3) My primary area of interest is in the field of gastroenterology and, in particular, inflammatory bowel diseases.

I have authored more than 150 publications, including more than 130 publications in peer reviewed journals.

4) I am a founder, major shareholder, and member of the Board of Directors of Prometheus, Inc., the licensee of the above-identified application.

5) I understand that the above-identified application has claims directed to methods of optimizing therapeutic efficacy and/or reducing toxicity by determining levels of 6-mercaptopurine metabolites. I understand that the claims stand rejected, in part, as allegedly obvious.

6) I understand that the above-identified application was filed on April 8, 1999, claiming the benefit of provisional application serial No. 60/101,714, filed on September 24, 1998.

7) Around the time the application was filed, I believe that there was skepticism of experts in the field of gastroenterology and inflammatory bowel disease that the levels of the 6-mercaptopurine metabolite 6-thioguanine specifically recited in the claims would be predictive of therapeutic efficacy or reduced toxicity. I am aware of at least two experts in the field of inflammatory bowel disease, William J. Sandborn, M.D., of the Mayo Clinic, Rochester, Minnesota, and Stephen B. Hanauer, M.D., of the University of Chicago Department of Medicine, Chicago, Illinois, who expressed skepticism that the 6-mercaptopurine metabolite levels specifically recited in the claims would be predictive of therapeutic efficacy.

8) I am a co-author with Dr. Sandborn of the reference Sandborn et al., Gastroenterology 117:527-535 (1999)(Exhibit A), which described a study of the effectiveness of intravenous administration of azathioprine for time of response. This reference was submitted for review in January of 1999 and was published in September of 1999.

9) The study described in Sandborn et al. was directed to determining the effectiveness of a loading dose of azathioprine administered intravenously on the time to response in patients with steroid-treated Crohn's disease who were beginning azathioprine therapy. The primary outcome measured was the rate of complete remission, defined as a Crohn's Disease Activity Index (CDAI) score <150 points and total steroid withdrawal at week 8. The intravenous administration of azathioprine did not decrease the time to response in the patients. It was also found that the rate of complete remission (CDAI <150 and total steroid withdrawal) was not significantly greater in patients with 6-thioguanine nucleotide (6TGN) concentrations ≥200 pmol/$8x10^8$ red blood cells (RBCs) (see page 531, first column, last sentence; and page 533, second column, paragraph bridging pages 533-534).

10) Regarding reducing toxicity, I have encountered skepticism by experts in the field that the recited levels of 6-thioguanine or 6-methyl-mercaptopurine are predictive of reducing toxicity.

11) In conclusion, I believe that around the time of filing of the above-identified application and its parent

application, April 8, 1999, and September 24, 1998, respectively, there was skepticism of experts in the field of gastroenterology and inflammatory bowel disease that the specifically recited levels of 6-meraptopurine metabolite would have been predictive of therapeutic efficacy or reduced toxicity.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

6-28-01
Date

Stephan R. Targan, M.D.

EXHIBIT 1: **Executed Declaration Pursuant to 37 C.F.R. §1.132**

Attorney Docket No.: P-PM 3474

Serial No.: 09/288,344

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on July 2, 2001.

By Deborah L. Cadena
Deborah L. Cadena, Reg. No. 44,048

July 2, 2001
Date of Signature

Exhibit A

ALIMENTARY TRACT

Lack of Effect of Intravenous Administration on Time to Respond to Azathioprine for Steroid-Treated Crohn's Disease

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Background & Aims: Azathioprine is effective for Crohn's disease but acts slowly. A loading dose may decrease the time to response. *Methods:* A placebo-controlled study was conducted in patients with active Crohn's disease despite prednisone treatment. Patients were randomized to a 36-hour infusion of azathioprine, 40 mg/kg (51 patients), or placebo (45 patients) followed by oral azathioprine, 2 mg/kg, for 16 weeks. Prednisone was tapered over 5 weeks. The primary outcome measure was complete remission at week 8, defined by discontinuation of prednisone and a Crohn's Disease Activity Index of ≤150 points. Erythrocyte concentrations of the azathioprine active metabolite, 6-thioguanine nucleotide, were measured. *Results:* At week 8, 13 patients (25%) were in complete remission in the azathioprine-loaded group compared with 11 patients (24%) in the placebo group. The frequency of complete remission did not increase after 8 weeks in either group. Both groups achieved steady state of 6-thioguanine nucleotide by week 2, and no differences were found in mean concentrations between the groups. There were no significant differences in the frequency of adverse events between the groups. *Conclusions:* A loading dose does not decrease the time to response in patients with steroid-treated Crohn's disease beginning azathioprine therapy. Steady state of erythrocyte 6-thioguanine nucleotide and complete response occurred earlier than previously reported.

In the treatment of Crohn's disease, 6-mercaptopurine (6MP) and its prodrug azathioprine (AZA) are efficacious.[1–8] However, widespread use of these medications is limited by the perception that they have a slow onset of action. Present et al.[4] reported that the mean time to response in patients with Crohn's disease treated with 6MP was 3.1 months and that 19% of responders required 4 months or more of treatment before improvement was observed. In a meta-analysis of all placebo-controlled trials of AZA/6MP for Crohn's disease, Pearson et al.[8] reported that a consistent clinical benefit for AZA/6MP therapy was observed only after 17 weeks or more of therapy. The reason that AZA/6MP may act slowly is unclear. Some small pharmacokinetic studies have reported that the active metabolites of AZA/6MP, the 6-thioguanine nucleotides (6TGN), have a long half-life resulting in slow accumulation in red blood cells (RBCs) and other body tissues and the need for prolonged treatment to reach steady state.[9,10] Other studies have reported that RBC 6TGN steady state is reached within 14–21 days in most patients.[11,12] These pharmacological observations raised the possibility that administration of a loading dose of AZA or 6MP could accelerate their onset of action. An open-label pilot study reported that patients with refractory Crohn's disease treated with an intravenous (IV) loading dose of AZA (1800 mg [20–44 mg/kg] over 36 hours) achieved a steady state of RBC 6TGN within 3 days and experienced rapid clinical

Abbreviations used in this paper: AZA, azathioprine; IBDQ, Inflammatory Bowel Disease Questionnaire; 6MP, 6-mercaptopurine; 6TGN, 6-thioguanine nucleotides; TPMT, thiopurine methyltransferase; WBC, white blood cell.

0016-5085/99/$10.00

EXHIBIT A

benefit.[13] Based on these preliminary results, we conducted a 16-week placebo-controlled trial of an IV AZA loading dose of 40 mg/kg in patients with active steroid-treated Crohn's disease beginning oral AZA treatment.

Patients and Methods

Selection of Patients

The study was performed between September 1996 and November 1997. Eligible patients were at least 18 years of age and had active steroid-treated Crohn's disease, as defined by a score of 150–450 on the Crohn's Disease Activity Index (CDAI) and treatment with prednisone at a dose of ≥20 mg/day for ≥4 weeks. The CDAI assesses 8 variables: the number of liquid stools, extent of abdominal pain, general well-being, occurrence of extraintestinal symptoms, need for antidiarrheal drugs, presence of abdominal masses, hematocrit, and body weight.[14] Scores can range from 0 (no active disease) to approximately 600 (severe disease). Scores of 150–450 are associated with disease that is mildly to moderately active. Scores of <150 indicate remission.

Eligible patients had disease that involved the ileum, ileocolon, or the colon, verified previously by colonoscopy, barium enema, or small bowel follow-through. Patients with currently present fistulas involving adjacent loops of bowel (enteroenteric fistulas) or the perianal region were eligible. In addition, patients had to have normal metabolism of AZA as defined by normal activity of the major catabolic enzyme for AZA, thiopurine methyltransferase (TPMT).[15] The following patients were not eligible: those with active Crohn's disease isolated to the duodenum, jejunum, or perianal region; those with currently present ileostomy or colostomy, septic complications, abscess, perforation with acute abdomen, or fistulas involving the skin, bladder, or vagina; those who had a stricture of the ileum or colon resulting in symptomatic obstruction (confirmed by endoscopic or radiological studies) within 6 months; those who had undergone resection of more than 100 cm of the ileum; or those requiring immediate surgery. Before the study, no patient received biotechnology therapies within 6 months, immune modifier drugs within 3 months, or antibiotics or mesalamine within 2 weeks, similar to the criteria used in other controlled trials of medical therapy for active Crohn's disease.[16–18] Patients with a history of cancer of any type, definite dysplasia of the colon within 5 years, or clinically significant renal or hepatic disease were ineligible, as were pregnant or breast-feeding women, patients who were allergic to 6MP or AZA, and patients receiving allopurinol. The study was approved by the institutional review board at each center, and all participants gave written informed consent.

Study Medication

The IV AZA loading dose of 40 mg/kg over 36 hours was chosen based on the preliminary data from a pilot study showing that an IV AZA loading dose of 20–44 mg/kg was safe and of apparent benefit[13] and data from phase I studies of continuous infusion 6MP for chemotherapy that showed significant toxicity with 6MP doses of 50 mg · m^2 · h^{-1} for 48 and 60 hours, and minimal toxicity with doses of 50 mg · m^2 · h^{-1} for 12, 24, and 36 hours.[19,20] Assuming a body surface of 1.73 m^2 and a body weight of 70 kg for the average adult, and a conversion factor of 2.07 for comparing 6MP with AZA (given 88% conversion of AZA to 6MP, and a molecular weight ratio of 0.55), 6MP infusions at 50 mg · m^2 · h^{-1} for 12, 24, 36, 48, and 60 hours would be comparable with 31, 61, 92, 123, and 153 mg/kg. Thus, the AZA loading dose of 40 mg/kg used in the present study is comparable with the 6MP dose of 50 mg · m^2 · h^{-1} for 12–24 hours, at which only minimal toxicity occurred.

Unblinded study pharmacists at each center prepared the IV infusions (AZA or placebo). For patients treated with AZA, the pharmacist determined the total amount of AZA to be administered (based on a total AZA dose of 40 mg/kg), divided the total amount by 3, reconstituted the appropriate number of 100-mg vials of AZA for injection (Imuran; Glaxo Wellcome, Research Triangle Park, NC), added the appropriate amount of reconstituted AZA into an empty 1000-mL Viaflex bag (Baxter Medical, Round Lake, IL), and added normal saline to obtain a total infusion volume of 600 mL. For patients treated with placebo, the pharmacist added 3 mL of Multi-Vitamins for Infusion (Schein Pharmaceuticals, Inc., Florham, NJ) and 597 mL of normal saline into an empty 1000-mL Viaflex bag (Baxter Medical). Patients then received 3 consecutive 600-mL infusions of study medication (AZA or placebo), each administered continuously at a rate of 50 mL/h for 12 hours. Both reconstituted AZA and multivitamins had a similar yellowish appearance designed to blind patients, investigators, and medical personnel caring for the patient to the treatment allocation. To further maintain blinding, the study pharmacist applied opaque sleeves to the infusion bags.

The oral AZA formulation used is a scored tablet containing 50 mg of AZA (Imuran, Glaxo Wellcome). The total daily dose of oral AZA was based on body weight (2.0 mg/kg), rounded upward to the nearest 25 mg. This dose of oral AZA, which was at the lower end of the range of AZA doses previously reported to be effective for Crohn's disease,[8] was chosen to give a margin of safety in patients who had just completed an IV loading dose of AZA of 40 mg/kg. The entire dose of AZA, 2.0 mg/kg, was administered as a single daily dose beginning on day 3 of the study. If patients developed laboratory abnormalities consistent with hepatotoxicity (levels of aspartate aminotransferase > 5× normal or alkaline phosphatase > 3× normal), leukopenia (total white blood cell count < 3.0 × 10^9/L), or thrombocytopenia (platelet count < 100 × 10^9/L), then AZA administration was discontinued until the abnormality resolved, and AZA was restarted at 1.0 mg · kg^{-1} · day^{-1}. Decisions regarding AZA discontinuation or dose adjustments in patients who experienced infection, fever, rash, arthralgias, malaise, and nausea were handled on a case-by-case basis. If patients developed pancreatitis or malignancy, AZA administration was discontinued. Compliance with oral AZA therapy was determined by pill count.

Prednisone Therapy

At the time of enrollment into the study (2 weeks before randomization), all patients had their prednisone dose adjusted to 20 mg/day, similar to the method reported previously by Feagan.[21] Thus, patients taking prednisone at a dose > 20 mg/day had their dose immediately reduced to 20 mg/day (regardless of the duration of steroid therapy or the duration of time at the current steroid dose); and patients taking 20 mg prednisone daily continued at that dose. For 2 weeks after randomization, no attempt was made to decrease the prednisone dose. After the first follow-up visit (at week 2), the daily prednisone dose was decreased by 5 mg each week. Prednisone was discontinued at the beginning of week 6. In subjects whose condition worsened (increase in the CDAI > 100 points above baseline or CDAI > 450), the prednisone dose was increased to 40 mg/day for 1 week and decreased by 5 mg each week until prednisone was discontinued.

Design of the Study

The study was a randomized, double-blind, placebo-controlled trial performed at 16 centers in the United States and Canada. Patients were stratified according to treatment center and randomized separately in permuted blocks of 2 by the unblinded study pharmacist at each center using a computer-generated randomization scheme. The blinded 36-hour infusion of 40 mg/kg AZA or placebo was followed by 16 weeks of open therapy with oral AZA, 2.0 mg · kg^{-1} · day^{-1}. The study duration of 18 weeks (2-week period of equilibration after standardization of prednisone and withdrawal of mesalamine and antibiotics, and then 16 weeks of AZA therapy) was based on the hypothesis that an IV AZA loading dose would accelerate the onset of action of AZA (making a long-term trial unnecessary) and on a meta-analysis of previous studies of oral AZA for active Crohn's disease, which showed that the odds ratio for response became significant with a study duration of 17 weeks.[8]

At entry, each patient's demographic characteristics, medical history, and current medications were recorded. Disease activity was assessed before treatment (after 2 weeks of screening observation); after 2, 4, 8, 12, and 16 weeks of treatment; and in the event that treatment was discontinued. Efficacy was not evaluated after the discontinuation of treatment in patients who withdrew from the study before week 16. At each visit, a physical examination, quality-of-life assessment, laboratory tests, and a global evaluation were conducted, and patients were asked whether any adverse events had occurred. No medications for Crohn's disease other than prednisone, the study drug, and antidiarrheals (loperamide, diphenoxylate, or opiates) were allowed.

Quality of life was assessed with the self-administered Inflammatory Bowel Disease Questionnaire (IBDQ), a previously validated instrument with 4 parts (bowel function, emotional status, systemic symptoms, and social function); the total score on this index ranges from 32 to 224, with higher scores indicating better quality of life. The scores of patients in remission usually range from 170 to 190.[22]

Patients recorded on diary cards their intake of study medication and prednisone, the frequency of loose stools, the extent of their abdominal pain, and general well-being during the 7 days before each visit. Blood samples were taken for hematologic and biochemical assessments and liver function tests. Blood was drawn for measurement of RBC TPMT enzyme activity at the screening visit (Mayo Medical Laboratories, Rochester, MN). The activity of the TPMT enzyme is subject to significant genetic variation.[15] There is a trimodal distribution of TPMT activity in the general population: low TPMT activity (<5.0 U/mL RBC) occurs at a frequency of 0.3%; intermediate TPMT activity (5.0–13.7 U/mL RBC) occurs at a frequency of 11.1%; and high or normal TPMT activity (13.8–25.1 U/mL RBC) occurs at a frequency of 88.6%.[15] Blood was also drawn for measurement of the active metabolite of AZA, 6TGN, in RBCs. The total RBC 6TGN concentration is based on the conversion of 6TGN to the free 6TGN base, which is then oxidized to a fluorescent sulfonate and assayed by high-performance liquid chromatography.[13,23] The intracellular 6TGN concentration thus represents the total RBC 6TGN contents consisting of a composite of the mono-, di-, and triphosphate nucleotides. The RBC 6TGN concentration is normalized to 8×10^8 RBCs. The assay is sensitive to 30 pmol with an overall interday coefficient of variance of 5%, as described previously.[13] The therapeutic range for RBC 6TGN in patients treated with AZA is unknown.

All adverse events were recorded, whether or not they were related to the study medication. A serious adverse event was defined as one that was life threatening or led to permanent disability, hospitalization, or death. The intensity of adverse events was graded as mild, moderate, or severe, with a severe event considered to be one that was incapacitating, leading to an inability to work or take part in normal activities.

Statistical Analysis

We estimated that 36 patients were needed in each group to detect an absolute difference of 35% between groups in the proportion of patients in complete remission, assuming complete remission rates of 60% with IV AZA treatment compared with 25% with standard oral AZA, and 80% power. We planned to recruit a total of 90 patients to allow for a dropout rate of up to 20%.

The primary outcome was the rate of complete remission, defined as a CDAI score < 150 points and total steroid withdrawal at week 8. The primary intention-to-treat population (as stated in the study protocol) included all patients who received at least one dose of study medication. Secondary end points were clinical remission (defined as a CDAI score < 150 points), clinical improvement (defined as a CDAI score < 150 points or total steroid withdrawal), clinical improvement (defined as a decrease in baseline CDAI ≥ 70 points), mean daily prednisone dose, mean CDAI scores, mean IBDQ scores, mean RBC 6TGN concentrations, mean white blood cell (WBC) concentrations, and adverse events.

The rates of complete remission were compared using a Mantel–Haenszel χ^2 test controlling for geographic cluster.

Clusters were used to ensure adequate representation across treatment groups and to minimize disproportionate influence by any given center. Comparisons of more than 2 rates were made using an extended Mantel–Haenszel test. Clinical remission and adverse event rates were compared using the Fisher exact test. A van Elteren test was use to compare the medians for CDAI scores, IBDQ scores, daily prednisone use, RBC 6TGN concentrations, and WBC concentrations. All tests were two-sided. P values < 0.05 were considered to indicate statistical significance.

For analysis of remission rates at 8, 12, and 16 weeks, we divided the number of patients in the group who were evaluated and in remission at that time, or who had already been withdrawn from the study while in remission, by the number of patients who were evaluated at that time or who had withdrawn before that time (last observation carried forward principle). The secondary end points of clinical remission and the 2 definitions of clinical improvement at 2, 4, 8, 12, and 16 weeks were analyzed similarly. In the analysis of RBC 6TGN and WBC concentrations, data on patients who were lost to follow-up or withdrawn from the study because of deterioration in their condition or adverse events were censored at the time of the last study visit.

Results

A total of 96 patients were enrolled; 51 were randomly assigned to receive IV AZA and 45 were assigned to receive placebo. All patients received the IV study medication and were included in the intention-to-treat analysis. The baseline characteristics of the 2 groups of patients were similar (Table 1). Thirty-two patients (63%) in the IV AZA group and 26 patients (58%) in the placebo group ($P = 0.679$) completed the scheduled 16 weeks of treatment and follow-up. Worsening Crohn's disease led to withdrawal of 5 patients in the IV AZA group and 4 patients in the placebo group. Adverse events led to the withdrawal of 8 patients in the IV AZA group and 12 patients in the placebo group. One patient in the IV AZA group declined to continue. Protocol violations led to withdrawal of 3 patients in the IV AZA group and 1 patient in the placebo group. In both groups, 2 patients were withdrawn for other reasons. The median compliance for oral AZA therapy was 96% in the IV AZA group and 91% in the placebo group.

Clinical Efficacy

The rates of complete remission were similar in the IV AZA and placebo groups throughout the study. The respective rates were 25% and 24% ($P = 0.906$) after 8 weeks, 22% and 22% ($P = 0.939$) after 12 weeks, and 31% and 27% ($P = 0.615$) after 16 weeks (Figure 1). Other measures of response including clinical remission and the 2 types of clinical improvement were also similar in the IV AZA and placebo groups throughout the study. The respective rates of clinical remission (CDAI < 150), clinical improvement (CDAI < 150 or total steroid withdrawal), and clinical improvement (decrease in baseline CDAI ≥ 70 points) after 2, 4, 8, 12, and 16 weeks are shown in Figure 1. The median time to first clinical remission (CDAI < 150) was identical in the IV AZA and placebo groups (28 vs. 28 days; $P = 0.984$).

The median scores on the CDAI, the IBDQ quality of life index, and the median daily prednisone doses were similar in the IV AZA and placebo groups throughout the 16-week study (Figure 2). The rate of complete remission (CDAI < 150 and total steroid withdrawal) was not significantly greater in patients in either treatment group who had a shorter duration of steroid therapy ($\leq$90 days) than in patients who had a longer duration of steroid therapy ($\geq$91 days). The IV AZA and placebo groups were similar with respect to the percentage of patients who had previously been treated with AZA/6MP (27% vs. 16%; $P = 0.179$), cyclosporine (8% vs. 7%; $P = 0.813$), and methotrexate (21% vs. 13%; $P = 0.410$). There were no significant differences in the rates

Table 1. Baseline Characteristics of the Patients

Variable	Placebo group (n = 45)	IV AZA group (n = 51)
Sex (M/F)	25/20	24/27
Age at entry (*yr*)		
Median	35	33
Range	19–65	19–63
Weight (*kg*)		
Median	68	78
Range	46–110	46–144
Duration of disease (*yr*)		
Median	6.6	7.1
Range	0–35	0–28
Duration of current exacerbation (*mo*)		
Median	3.2	3.8
Range	0–16	1–51
CDAI		
Median score	245	244
Range	142–476	89–424
IBDQ		
Median score	123	127
Range	77–182	73–183
TPMT activity (*U/mL*)		
Median	17.9	19.8
Range	14–36	14–28
Disease site (no. of patients)		
Ileum	8	17
Ileocolon	27	27
Colon	10	7
Previous intestinal resection (no. of patients)	26	16
Cigarette smoker (no. of patients)	16	22
Duration of steroid use (*days*)		
Median	70	70
Range	29–7300	3–330

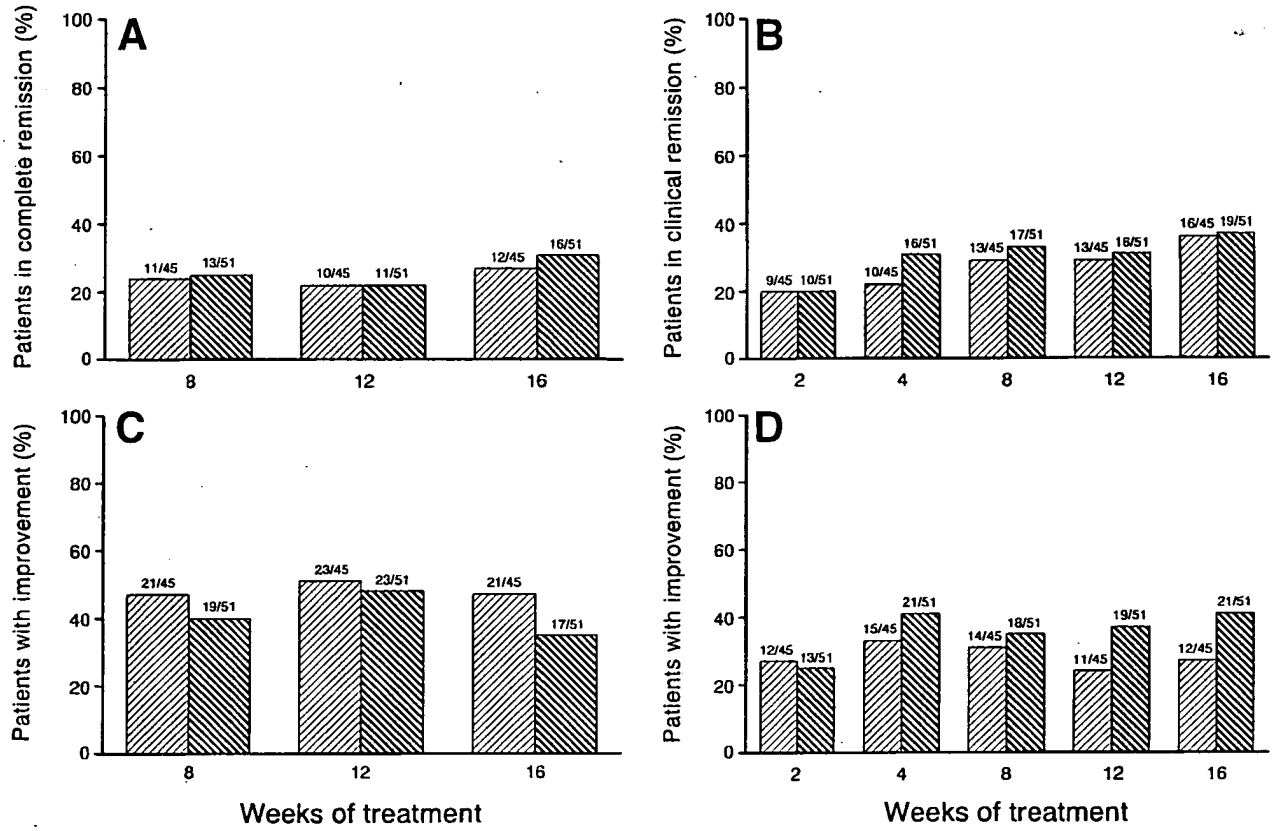

Figure 1. Percentages of patients with Crohn's disease with improvement or remission at each study visit, according to treatment group. ▨, Placebo; ▩, IV AZA. There were no significant differences between the 2 groups at any time point. (*A*) Complete remission (CDAI score < 150 points and total steroid withdrawal). (*B*) Clinical remission (CDAI score < 150 points). (*C*) Improvement (CDAI score < 150 points or total steroid withdrawal). (*D*) Improvement (decrease in CDAI score from baseline ≥ 70 points).

of complete remission in the IV AZA and placebo groups when stratified according to previous therapy with AZA/6MP, cyclosporine, or methotrexate.

Azathioprine Dose, 6TGN Concentrations, TPMT Activity, and WBC Concentrations

The median baseline RBC TPMT activities were similar in the IV AZA and placebo groups (Table 1). The median RBC 6TGN concentrations were significantly greater at the end of the 36-hour infusion (week 0.2) and at week 1 in the IV AZA group but were similar at all other time points throughout the 16-week study (Figure 2). The overall mean daily doses of oral AZA in the IV AZA and placebo groups (2.00 ± 0.31 and 2.15 ± 0.24 mg/kg, respectively) were associated with overall mean RBC 6TGN concentrations using within subject means of 164 ± 61 and 124 ± 67 pmol/8 × 10^8 RBCs. The correlation between the baseline TPMT activities and the mean within subject RBC 6TGN concentrations over 16 weeks in the combined patient groups was $r = -0.0137$, $P = 0.8975$. The rate of complete remission (CDAI < 150 and total steroid withdrawal) was not significantly greater in patients with 6TGN concentrations of ≥200 pmol/8 × 10^8 RBCs.

The mean total WBC concentrations were significantly lower at weeks 1 and 2 in the IV AZA group than in the placebo group but were otherwise similar throughout the 16-week study (Figure 2). The correlation between the total WBC concentrations and the corresponding RBC 6TGN concentrations in each patient in the combined patient groups over weeks 1–16 was $r = -0.11$, $P = 0.30$. The rate of complete remission (CDAI < 150 and total steroid withdrawal) was not significantly greater in patients with mild leukopenia (defined as a total WBC concentration ≤ 5.0 × 10^8/L).

Adverse Events

The numbers of patients with adverse events were similar in the 2 groups, with the exception of nausea and infusion site reactions, which were more common in the IV AZA group; the most frequent adverse events and adverse events known to be associated with AZA are shown in Table 2. Serious adverse events and severe adverse events were also similar in both groups. Aside from the decrease in the mean total WBC concentrations in the IV AZA group at weeks 1 and 2 (discussed above), there were no clinically significant changes in any other hematologic or biochemical variables in either group.

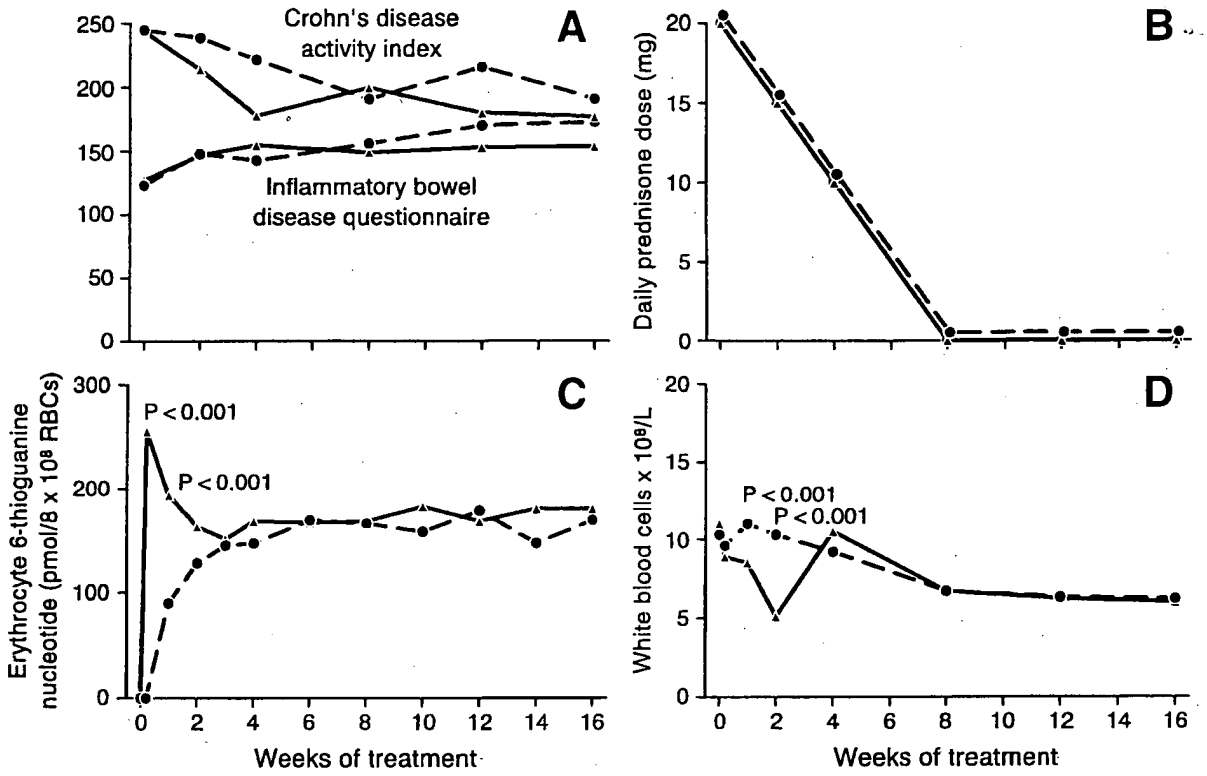

Figure 2. Median scores or values at each study visit according to treatment group. ●, Placebo; ▲, IV AZA. The only significant differences between the 2 groups at any of the time points are those indicated in the figure. (*A*) CDAI and IBDQ quality of life index. (*B*) Prednisone dose. (*C*) RBC 6TGN nucleotide concentrations. (*D*) Total WBC concentrations.

Table 2. Adverse Events in the Two Treatment Groups

Variable	Placebo group (n = 45)	IV AZA group (n = 51)	*P* value
No. of adverse events	185	258	0.730
No. of patients with adverse events	40 (89%)	47 (92%)	0.730
No. of patients with serious adverse events	11 (24%)	13 (25%)	1.000
No. of patients with severe adverse events	14 (31%)	19 (37%)	0.667
Most frequent adverse events (no. of events)			
Nausea	15 (33%)	28 (55%)	0.041
Injection site reaction	1 (2%)	11 (22%)	0.005
Headaches	10 (22%)	7 (14%)	0.298
Malaise and fatigue	9 (20%)	8 (16%)	0.604
Vomiting	8 (18%)	9 (18%)	1.000
Viral lower respiratory infection	6 (13%)	6 (12%)	1.000
Abdominal pain	5 (11%)	5 (10%)	1.000
Arthralgias	3 (7%)	5 (10%)	0.719
Adverse events commonly associated with AZA (no. of events)			
Leukopenia[a]	5 (11%)	5 (10%)	1.000
Pneumonia	2 (4%)	0 (0%)	0.217
Thrombocytopenia	0 (0%)	2 (4%)	0.497
Pancreatitis	2 (4%)	0 (0%)	0.497
Abnormal liver function tests	1 (2%)	0 (0%)	0.469
Fever	4 (9%)	4 (8%)	1.000
Rash	2 (4%)	3 (6%)	1.000

[a]Clinically significant leukopenia defined as a total WBC $<3.0 \times 10^8$/L.

Discussion

We found that administration of a loading dose of IV AZA does not decrease the time to response or increase the response rate in patients with active steroid-treated Crohn's disease beginning oral AZA treatment. The condition of patients in both groups improved after 2 weeks and reached a plateau after 4 weeks. The degree of response was similar in the 2 groups throughout the study, as measured by complete remission, clinical remission, and the 2 types of clinical improvement. Other measures of response, such as a decrease in mean daily prednisone dose and an increased quality of life, also showed a similar degree of improvement in the 2 groups throughout the study. The duration of prestudy steroid therapy had no effect on response. The relatively high rates of early withdrawal (37% for the IV AZA group and 42% for the placebo group) are similar to those in other studies of medical therapy for active Crohn's disease.[16–18,21] Given the size of this study and the comprehensive nature of the outcome measures, it is unlikely that a clinically important benefit went undetected. Based on these findings, we do not recommend the use of an IV loading dose of AZA in patients with Crohn's disease who are beginning oral AZA treatment.

One assumption that our study was based on is that AZA and 6MP have a slow onset of action. The results of

the study suggest that this assumption may be incorrect and that the evidence underlying this belief should be reexamined. The results from 3 placebo-controlled trials of oral AZA for active Crohn's disease published in the early 1970s can probably be dismissed as invalid because of methodological problems including small sample size, crossover design, and use of unvalidated end points.[1,24,25] In 1980, Present et al.[4] reported a placebo-controlled trial showing that oral 6MP was effective for active Crohn's disease. Although this important study established a role for 6MP/AZA in the treatment of Crohn's disease, methodological problems including a heterogenous patient population, crossover design, use of unvalidated end points, and infrequent assessments of clinical response make poststudy subgroup analyses and observations suspect. In particular, the authors' assertion that the mean time to response was 3.1 months may represent an overestimate because patients were only clinically assessed every 3 months. Two other placebo-controlled studies of oral AZA or 6MP failed to show efficacy at week 12 but did show efficacy at 12–15 months.[6,7] In these studies, initial combination therapy with high-dose corticosteroids, which was tapered slowly over 12 weeks or more, makes it difficult to determine the time to response for AZA/6MP. In the National Cooperative Crohn's Disease Study, monotherapy with oral AZA was compared with placebo.[26] There was a trend toward a benefit for AZA-treated patients that did not reach statistical significance. The maximum decrease in the mean CDAI score for the AZA-treated patients occurred at 9 weeks. In another study, oral AZA administered in combination with high-dose corticosteroids (which were rapidly tapered to 10 mg/day over 6 weeks) showed a significant benefit for AZA by week 8.[5] Finally, our study showed that patients in the placebo group (who were beginning standard oral AZA therapy) reached a plateau of the proportion of patients who experienced a clinical response after 4 weeks. These findings suggest that oral AZA or 6MP may act more rapidly than previously believed, perhaps over 4–8 weeks.

Without a placebo control group, it is difficult to directly compare the complete remission rates in our study (31% for the IV AZA group and 27% for the standard oral AZA group at 16 weeks) with those of other controlled trials of oral AZA for active Crohn's disease. Nevertheless, the remission rates in our study seem to be generally lower than those previously reported.[8] The likely explanation is that the patients entering our trial were steroid refractory and that we required complete steroid withdrawal as part of the definition of remission. The remission rates in our study seem similar to 2 other recent trials of methotrexate and infliximab for active Crohn's disease refractory to steroids and other medical therapies.[21,27]

Another assumption that our study was based on is that 6TGN accumulates slowly in RBCs and other body tissues, and prolonged treatment with oral AZA/6MP is required to reach steady state. The results of our study indicate that this assumption is incorrect. We showed that steady-state concentrations of RBC 6TGN were achieved by 2 weeks in patients in the placebo group who were beginning standard oral AZA treatment. Although the mean RBC 6TGN concentrations were significantly higher in the IV AZA group at weeks 0.2 and 1, they were similar beginning at week 2 for the remainder of the 16-week study. Our results corroborate the findings of 2 other pharmacokinetic studies in patients with acute lymphoblastic leukemia or renal allografts treated with 6MP or AZA in which RBC 6TGN concentrations were reported to reach steady state in 14–21 days.[11,12] These findings show that patients with active Crohn's disease treated with oral AZA reach steady-state concentrations of RBC TGN over 2–3 weeks.

Our study does not clarify the clinical use of measuring the activity of the major catabolic enzyme of AZA (RBC TPMT) before starting oral AZA therapy and of doing therapeutic drug monitoring with RBC 6TGN concentrations during oral AZA therapy. Patients were eligible for our study only if they had normal RBC TPMT activity, leading to a relatively homogenous and low-risk patient population. There was not a statistically significant inverse correlation between baseline RBC TPMT activity and mean within subject RBC 6TGN concentrations over 16 weeks. An inverse relationship would likely have been shown if patients with intermediate and homozygous low TPMT activity had been included, similar to previous reports in children with acute lymphoblastic leukemia[28] and adults with rheumatoid arthritis.[29] We did not find that RBC 6TGN concentrations of $\geq$200 pmol/8 $\times$ 10^8 RBCs were associated with a greater likelihood of response. These findings are in contrast to a prior report suggesting a correlation between clinical response and RBC 6TGN in patients with Crohn's disease treated with a range of 6MP doses.[30] The average RBC 6TGN concentrations over 16 weeks in our patients treated with IV placebo followed by 2 mg/kg oral AZA were relatively low (mean, 124 pmol/8 $\times$ 10^8 RBCs) compared with concentrations reported in patients with acute lymphoblastic leukemia treated with 75 mg/m^2 oral 6MP (median, 284 pmol/8 $\times$ 10^8 RBCs).[31] To further explore the potential use of RBC 6TGN therapeutic drug monitoring in patients treated with oral AZA, additional studies including patients with intermediate or homozygous low

TPMT activity and exploring a range of oral AZA doses (perhaps 0.5–4.0 mg/kg) should be undertaken.

There was a significant but transient decrease in the mean total WBC concentration in the IV AZA group at weeks 1 and 2; otherwise both study groups had a parallel decrease in the mean total WBC concentration that plateaued at week 8. Ten percent of patients in the IV AZA group developed clinically significant leukopenia (total WBC $< 3.0 \times 10^8$/L) during the study compared with 11% in the placebo group. The rate of leukopenia in the placebo group (standard oral AZA) is similar to the leukopenia rates of 2%–11% reported in other safety studies of AZA and 6MP in patients with IBD.[32-34] There was not a statistically significant inverse correlation between the total WBC concentrations and the corresponding RBC 6TGN concentrations over weeks 1–16. An inverse relationship would likely have been shown if patients with intermediate or homozygous low TPMT activity and a wider range of AZA doses had been included, similar to a previous report in children with acute lymphoblastic leukemia.[35] We were not able to identify a cutoff value for the RBC 6TGN concentration that accurately predicted leukopenia. A retrospective study suggested an association between mild leukopenia (defined as a total WBC concentration $\leq 5.0 \times 10^8$/L) and remission in patients with Crohn's disease treated with 6MP.[36] In our study, the rate of complete remission (CDAI < 150 and total steroid withdrawal) was not significantly greater in patients with mild leukopenia; we are therefore unable to confirm to the previous association.

AZA therapy was generally well tolerated in both patient groups, and the frequency and type of AZA-associated adverse events observed were similar to those reported in other studies.[32-34] The frequency of severe and serious adverse events (according to World Health Organization definitions) was relatively high, but generally comparable with other studies of medical therapy for active Crohn's disease.[17,18,21,27] The serious adverse events were all related to need for hospitalization, in most cases because of worsening of Crohn's disease. Our results show that pretreatment with an IV AZA loading dose before beginning oral AZA treatment, although not of clinical benefit, is as safe as standard treatment with oral AZA in patients with normal RBC TPMT activity.

In conclusion, a loading dose is safe but not effective in decreasing the time to response in patients with steroid-treated Crohn's disease beginning AZA treatment. Steady-state concentrations of RBC 6TGN and clinical improvement/remission in patients receiving standard oral AZA occurred earlier than previously reported. Additional studies to explore the time to response, the dose response, and the clinical use of therapeutic drug monitoring in patients with Crohn's disease treated with oral AZA are warranted.

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ORIGINAL ARTICLE

Therapeutic Drug Monitoring of Azathioprine and 6-Mercaptopurine Metabolites in Crohn Disease

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Belaiche J, Desager JP, Horsmans Y, Louis E. Therapeutic drug monitoring of azathioprine and 6-mercaptopurine metabolites in Crohn disease. Scand J Gastroenterol 2001;36:71–76.

Background: 6-Mercaptopurine (6-MP) and its prodrug azathioprine (AZA) have proven efficacy in the treatment of Crohn disease (CD). The immunosuppressive properties of AZA/6-MP are mediated by the intracellular metabolism of 6-MP into its active metabolites, 6-thioguanine nucleotides (6TGN) and 6-methylmercaptopurine (6-MMP). Preliminary studies have suggested that the red blood cell concentration of 6TGN (RBC 6TGN) is a potential guide to therapy. The aims of the study were to evaluate the RBC 6TGN concentrations in adult patients with CD under long-term AZA/6-MP therapy and to correlate it with response to treatment and haematological parameters. **Methods:** Twenty-eight CD patients treated for at least 3 months with AZA/6-MP were prospectively studied. Patients were separated into three main groups: group 1 ($n = 19$), corresponding to quiescent CD receiving AZA (dose: 2.05 ± 0.4 mg/kg/day for a mean of 28.6 ± 25 months) or 6-MP (dose: 1.4 ± 01 mg/kg/day for a mean of 7.5 ± 3.5 months) alone; group 2 ($n = 6$), corresponding to quiescent CD treated by AZA (dose: 2.14 ± 0.5 mg/kg/day for a mean of 29.5 ± 22 months) with oral steroids; and group 3 ($n = 3$), corresponding to active CD on AZA (dose: 1.94 ± 0.6 mg/kg/day for a mean of 31.3 ± 35 months) as the only treatment. An assessment was also made by merging groups 1 and 2 forming a larger group of patients ($n = 25$) defined by clinical remission and groups 2 and 3 forming a larger group of patients ($n = 9$), non-complete responders with AZA/6-MP alone. Crohn disease index activity (CDAI), blood samples for full blood count and differential white cell count and measurement of RBC 6TGN and 6-MMP concentrations were evaluated at inclusion and at 6 months ($n = 17$). RBC 6TGN were measured using high performance liquid chromatography (HPLC) on heparinized blood. **Results:** The baseline characteristics of the three groups of patients were similar. There was no significant difference among the three groups of patients regarding the dose and the duration of immunosuppressive treatment. There was no significant difference between groups according to various parameters tested. Particularly, the median RBC 6TGN concentration at inclusion was similar in the three groups of patients (166 (105–688), 183 (90–261) and 160 (52–194) pmol/8 × 10^8 RBC, respectively). The majority of patients had no detectable level of 6-MMP metabolite, except for 3 patients. There was also no difference between merging groups. Furthermore, there was no significant correlation between RBC 6TGN concentrations and the various biological parameters tested except for the mean erythrocyte volume. At 6 months, all patients of group 1 remained in remission and median RBC 6TGN concentration remained stable. No side effects were observed. **Conclusions:** There is, contrary to preliminary studies, a broad overlap in RBC 6TGN levels as well as for haematological parameters in patients in remission or not and responders or not to AZA/6-MP therapy. This suggests, beside a variability in the metabolism of these drugs, the existence of complex mechanisms of action. Nevertheless, beside the use of RBC 6TGN determination to confirm compliance to therapy, this dosage could be useful in non-responding patients, allowing, in absence of leukopenia, to increase the dose of AZA/6-MP safely.

Key words: Azathioprine; Crohn disease; 6-mercaptopurine; metabolism; monitoring

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6-Mercaptopurine (6-MP) and its prodrug azathioprine (AZA) have proven efficacy in the treatment of Crohn disease (CD). A meta-analysis of all placebo-controlled trials of AZA/6-MP treatment for CD reported overall response rates of 56% and 67% for treatment of active disease and remission maintenance indications (1). These drugs have now been recognized as having a major role in the treatment of steroid dependent CD, eliminating the need for corticosteroids in about 75% of patients, with a median response time of 3–4 months (2, 3). Furthermore, these immunosuppressants have also been shown to improve the quality of life (4). However, potential

EXHIBIT 2

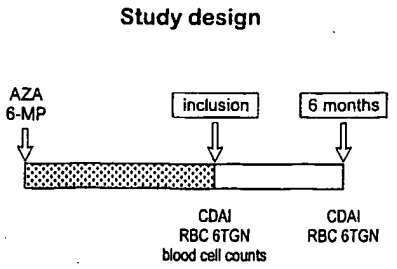

Fig. 1. Design of the study.

complications of treatment occur in 15% of patients (5). Moreover, 25%–35% of the patients do not respond to AZA/6-MP treatment even after several months, raising the possibility that altered drug metabolism effects efficacy. Therapeutic drug monitoring during treatment with AZA/6-MP has the potential to address these problems. The immunosuppressive properties of AZA/6-MP are considered to be mediated mainly by the intracellular metabolism into its active 6-thioguanine nucleotides (6TGN) (5). 6TGN have a long half-life resulting in slow accumulation in red blood cells (RBCs) and other body tissues and the need for prolonged treatment to reach steady-state levels. These metabolites seem thus to be appropriate candidates for such monitoring. However in CD, only a recently published paper in paediatric series of CD indicates that monitoring based on red blood cell concentration of 6TGN (RBC 6TGN) is a potential guide to therapy (6).

The aim of this study was to evaluate the RBC 6TGN concentrations in adult patients with CD on long-term therapy with AZA or 6-MP and to correlate it with response to treatment and haematological parameters.

Patients and Methods

Patients

The study was performed between March 1999 and December 1999. The study design is shown in Fig. 1. Eligible patients were all patients with CD seen during this period and treated for at least 3 months by AZA/6-MP. Twenty-eight patients with CD (19 females, 9 males), mean age 37 years (range, 24–58), were included in this study. The diagnosis of CD was made using the clinical, morphological and histological criteria of Gower-Rousseau et al. (7). The reason for immunosuppressive treatment had been in all cases corticosteroid dependent CD, except in 2 patients with an early severe postoperative recurrence. The corticosteroid dependency was defined as previously reported (8), either by two successive relapses during the 2 months following steroid discontinuation, or by two successive relapses at dose tapering after successful treatment of flare-up with steroids. The activity of CD was determined at inclusion by the Crohn Disease Activity Index (CDAI). Twenty-five patients had quiescent CD (CDAI < 150) and 3 patients had active disease (CDAI: median score, 210; range, 200–395) despite immunosuppressive treatment. The patients were separated into three main groups at inclusion (Table I). Patients with quiescent CD were separated into two groups: group 1 corresponding to 19 patients treated with AZA ($n = 17$) or 6-MP ($n = 2$) as the only treatment; group 2 corresponding to 6 patients treated by AZA and methylprednisolone (mean dose: 13 mg/day ± 10). Group 3 included the 3 patients with active symptomatic CD. These 3 patients were on AZA as the only treatment. The baseline characteristics of the three groups of patients are summarized in Table II. An assessment was also made by merging groups 1 and 2, forming a larger group of patients

Table I. Definition of the groups of patients at inclusion

Group 1	Quiescent CD with AZA/6-MP alone (complete responders)
Group 2	Quiescent CD with AZA + oral steroids
Group 3	Active CD under AZA as only treatment
Group 1 + 2	Patients in clinical remission
Group 2 + 3	Non-complete responders with AZA alone

Table II. Baseline characteristics of the patients at inclusion

Variable	Group 1 ($n = 19$)	Group 2 ($n = 6$)	Group 3 ($n = 3$)
Sex (M/F)	4/15	1/5	0/3
Age at entry (years) (mean ± s, range)	36.8 ± 10.4 (24–58)	43.6 ± 8.5 (37–58)	32 ± 3.5 (30–36)
Duration of disease (years) (mean ± s, range)	10.2 ± 5.9 (1–23)	12.6 ± 7.7 (2–22)	10.6 ± 2 (9–13)
Duration of remission (months) (median, range)	10 (2–84)	7.5 (3–24)	NA
Disease site (no. of patients)			
Ileum	2		
Ileocolon	10	1	2
Colon	7	5	1
Azathioprine*			
Duration (months) (mean ± s, range)	28.6 ± 25 (3–84)	29.5 ± 22 (3–60)	31.3 ± 35 (5–72)
Dose (mg/kg/day) (mean ± s, range)	2.05 ± 0.4 (1.35–2.8)	2.14 ± 0.5 (1.5–3)	1.94 ± 0.6 (1.88–2)
6-MP †			
Duration (months) (mean ± s, range)	7.5 ± 3.5 (5–10)		
Dose (mg/kg/day) (mean ± s, range)	1.4 ± 0.1 (1.35–1.5)		

NA = not applicable.
* $n = 17$.
† $n = 2$.

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Table III. Biological profiles of the patients at inclusion

Variable	Group 1 ($n = 19$)	Group 2 ($n = 6$)	Group 3 ($n = 3$)
RBC 6TGN (pmol/8 × 10^8 RBCs) (median, range)	166 (105–688)	183 (90–261)	160 (52–194)
MCV (μ^3) (mean ± s, range)	93 ± 8 (74–104)	96 ± 3.5 (90–100)	83 ± 7 (77–91)
Leukocyte count (×10^3/ml) (mean ± s, range)	6.4 ± 1.81 (3.34–9.17)	9.6 ± 2.7 (5.9–10.3)	5.9 ± 0.8 (5.1–6.7)
Neutrophils count (×10^3/ml) (mean ± s, range)	4.6 ± 1.1 (2.2–7.7)	7.1 ± 1.5 (5.1–8.6)	4.3 ± 1.1 (3.0–5.2)
Relative neutrophilia (%) (mean ± s, range)	67 ± 15 (15–84)	74 ± 7.5 (66–84)	71.5 ± 15 (59–78)
Lymphocyte count (×10^3/ml) (mean ± s, range)	10.8 ± 4.5 (6–22)	15.6 ± 5 (10–22)	9 ± 3.5 (5–11)
Relative lymphocytosis (%) (mean ± s, range)	17.8 ± 7.7 (6.2–33.8)	16 ± 5.6 (9.0–21)	15.9 ± 6.9 (8.7–22.5)

($n = 25$) defined by clinical remission, and groups 2 and 3, forming a larger group of patients ($n = 9$), non-complete responders with AZA or 6-MP alone (Table I).

Study variables

In addition to clinical assessment, blood samples were taken for total full blood count and differential white cell count and measurement of RBC 6TGN concentrations. The total RBC 6TGN concentration measurement was based on the conversion of 6TGN to the free 6-thioguanine base, assayed by high performance liquid chromatography (HPLC) on heparinized blood according to the method of Lennard & Singleton (9). Two quality control samples at 119 and 299 pmol/8 × 10^8 were analysed during each run. The intra-day coefficients of variation were 4.1% (110 ± 4.6 pmol/8 × 10^8 RBCs) and 4.5% (304 ± 13.7 pmol/8 × 10^8 RBCs), respectively, for $n = 6$. The interday coefficients of variation were 8% (120 ± 9.5 pmol/8 × 10^8 RBCs) and 6.9% (300 ± 20.8 pmol/8 × 10^8 RBCs), respectively, for $n = 17$. The sensitivity for 6TGN reached 5 pmol/8 × 10^8 RBCs. The '6-methylmercaptopurine like' (6-MMP) metabolite was detected on the same chromatogram with a sensitivity of 1 nmol/8 × 10^8 RBCs.

The assessment was performed firstly by comparing RBC 6TGN concentrations and haematological parameters between groups. Secondly, according to previous published RBC 6TGN data, we tried to validate a discriminant RBC 6TGN level by comparing the response to treatment in subgroups of patients divided according to the RBC 6TGN levels (230 pmol/8 × 10^8 RBCs (10), 250 pmol/8 × 10^8 RBCs (11)).

Seventeen patients of 28 included (group 1 = 10; group 2 = 4; group 3 = 3) were re-evaluated at 6 months for disease activity and RBC 6TGN concentrations.

Table IV. Correlations (r) between RBC 6TGN concentrations, treatment and haematological parameters at inclusion

Variable	r	P
AZA/6-MP dose (mg/kg/day)	0.020	0.917
AZA/6-MP duration	0.060	0.758
Leukocyte count	0.243	0.241
Neutrophil count	0.180	0.387
Relative neutrophilia	0.168	0.42
Lymphocyte count	−0.029	0.889
Relative lymphocytosis	−0.332	0.104
MCV	0.375	0.048

Statistical analysis

Comparative statistics were carried out using Student t test or Mann–Whitney U test as appropriate. Correlations between the various parameters were assessed by Spearman test. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

The baseline characteristics of the three patient groups were similar (Table II). There was no significant difference among the three groups of patients regarding the dose and duration of immunosuppressive treatment.

There was no significant difference between groups according to various parameters tested (Table III). Particularly, the median RBC 6TGN concentrations at inclusion are similar in the three groups of patients. There was also no difference between group 1 and group 2 + 3 (166 pmol/8 × 10^8 RBCs (range, 105–688) and 167 pmol/8 × 10^8 RBCs (range, 52–261), respectively), and between group 1 + 2 and group 3 (167 pmol/8 × 10^8 RBCs (range, 90–688) and 160 pmol/8 × 10^8 RBCs (range, 52–194), respectively). Furthermore, there was no significant correlation between RBC 6TGN concentrations and the various biological parameters tested except for the mean erythrocyte volume (MCV) which was higher in patients on AZA (Table IV).

The number of patients having a RBC 6TGN level higher than the two discriminant levels proposed in the literature were 6 and 8 for 250 and 230 pmol/8 × 10^8 RBCs, respectively. The proportion of patients as complete or non-complete responders on AZA/6-MP, according to these discriminant levels, are shown in Table V. No significant differences were found between patients above or below the discriminant levels. A low level of 6-MMP was noted in 2 patients of group 1 and 1 patient of group 2 (8.5, 6.0, and 4.2 nmol/8 × 10^8 RBCs, respectively).

At 6 months, all patients in group 1 ($n = 10$) remained in remission (CDAI < 150) with no modification (except 1 patient, see below) of AZA/6-MP therapy. RBC 6TGN levels remained relatively stable except in the patient with the highest concentration at inclusion (688 pmol/8 × 10^8 RBCs)

Table V. Proportion of patient responders and non-complete responders according to the two RBC 6TGN discriminant levels proposed in the literature (10, 11)

Patients	RBC 6TGN > 250 pmol/8 × 10^8 RBCs	RBC 6TGN < 250 pmol/8 × 10^8 RBCs	RBC 6TGN > 230 pmol/8 × 10^8 RBCs	RBC 6TGN < 230 pmol/8 × 10^8 RBCs
Complete responders (group 1 = 19)	5/6	14/22	6/8	13/20
Non-complete responders (group 2 + 3 = 9)	1/6	8/22	2/8	7/20

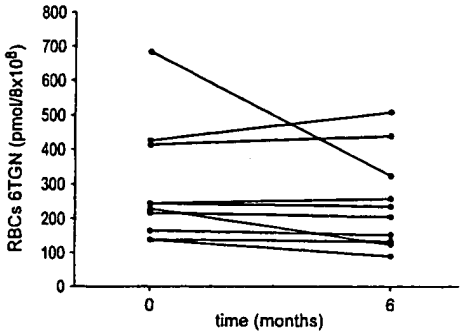

Fig. 2. RBC 6TGN concentrations at inclusion and at 6 months in quiescent CD treated by AZA/6-MP alone (patients $n = 10$ of group 1). The RBC 6TGN levels remained relatively stable. The median (range) coefficient of variation over this 6 months period was 4% (3.6%–40.7%).

in whom the dose of AZA had to be decreased (Fig. 2). The median (range) coefficient of variation over this 6-month period was 4% (3.6%–40.7%). In group 2 ($n = 4$), 1 patient had a flare-up at 5 months. At this time the level of RBC 6TGN concentration had decreased from 235 to 103 pmol/8 × 10^8 RBCs, despite the absence of treatment modification. The other patients were still in remission with the same dose of AZA and 2 of them were weaned from steroids (Table VI). The follow up of the 3 patients in group 3 is shown on Table VII.

No side effects were observed.

Discussion

We evaluated in the present study the monitoring of long-term treatment with AZA/6-MP by RBC 6TGN concentrations in patients with CD. The data showed a broad overlap in RBC 6TGN concentrations as well as haematological parameters in patients in remission or not and responders or not to AZA/6-MP. Accordingly, in this relatively small population, we could not confirm the previously proposed discriminant RBC 6TGN levels between responders and non-responders to AZA/6-MP.

AZA/6-MP are immunosuppressive drugs that are effective in the treatment of inflammatory bowel disease. These drugs have now been recognized as major drugs in the treatment of steroid dependent and chronic active CD. A recently published meta-analysis of the therapeutic trials of AZA in CD showed a significant efficacy both in active disease and in maintenance of remission (1). In these two situations, the efficacy correlated with the cumulative dose administered, depending on the dose and duration of the treatment. When using an appropriate dose, which is at least 2 mg/kg/day for AZA and 1.5 mg/kg/day for 6-MP, and waiting enough time for judgement of efficacy (at least 3–4 months), 60%–70% of patients show a significant clinical response while 25%–35% of patients do not respond even after several months. In a recent pilot study of 18 non-responders after a mean time of 11 months at a mean dose of 2 mg/kg/day, the increase of AZA to 2.5–3 mg/kg/day brought up a significant clinical response in 78% of the patients (12). In that study, the increase in AZA dosage was decided empirically, simply checking that it was not associated with major leukopenia. A previous study with 6-MP in refractory CD had showed that the clinical response correlated with the development of a relative leukopenia and neutropenia (below 5000 leukocytes/ml) (13). However, in another well-conducted recent prospective study, this correlation was not confirmed (14). Furthermore, the concentration of 6-MP itself seems to be of no value in monitoring the treatment, probably because this substance is inactive and has a very short half-life (1–2 h) in plasma (15). The considerable variability in optimal dosage of

Table VI. Follow up at 6 months for patients of group 2

Patient	RBC 6TGN at inclusion (pmol/8 × 10^8)	RBC 6TGN at 6 months (pmol/8 × 10^8)	Comments
MB	261	190	Steroid withdrawal at 3 months
JPL	235	NA*	Flare-up at 5 months treated by anti-TNF
LP	200	224	Steroid withdrawal at 2 months
ND	100	139	Quiescent CD with same dose of steroid

* Not available (RBC 6TGN at time of flare-up (5 months): 103 pmol/8 × 10^8).

Table VII. Follow up at 6 months for patients of group 3

Patient	RBC 6TGN (pmol/8 × 10^8 RBCs) at inclusion	RBC 6TGN (pmol/8 × 10^8 RBCs) at 6 months	Comments
HT	52	200	AZA increased from 1.8 to 2.5 mg/kg/day and ciprofloxacin added at inclusion. In remission 2 months later with AZA alone
NP	160	95	AZA increased from 2.5 to 3 mg/kg/day and oral budesonide added at inclusion. In remission 6 months later with AZA alone
JF	194	235	AZA increased from 1.6 to 2 mg/kg/day and methylprednisolone added at inclusion. In remission 6 months later with AZA alone

6-MP is probably related to the large variability in its metabolization by the three major pathways. After rapid non-enzymatic conversion of AZA to 6-MP, three enzymes compete to metabolize 6-MP: xanthine oxidase, which converts 6-MP to the inactive metabolite 6-thiouracic; thiopurine methyltransferase (TPMT), which converts 6-MP to 6-MMP (unclear metabolic activity); and hypoxanthine phosphoribosyl transferase followed by inosine monophosphate dehydrogenase and guanosine monophosphate synthetase, which convert 6-MP to the active metabolites 6TGN. Thus, RBC 6TGN concentration appears to be a potential candidate for the monitoring of therapeutic efficacy of AZA/6-MP. Pharmacological studies showing a prolonged half-life and a slow accumulation of 6TGN in the erythrocyte have been challenged by a recent study demonstrating that a steady state of RBC 6TGN concentration was obtained after only 2 weeks, even following classical oral treatment (14).

The RBC 6TGN concentration monitoring in patients treated with AZA/6-MP has been used in leukaemia and transplantation (16–19). In the treatment of children with acute lymphoblastic leukaemia treated with 6-MP, the RBC 6TGN concentration associated with efficacy was greater than 275 pmol/8 × 10^8 RBCs (16) while significant leukopenia was induced at concentration higher than 1000 pmol/8 × 10^8 RBCs (17). Therefore, the therapeutic window for RBC 6TGN in leukaemia seems to be 275–1000 pmol/8 × 10^8 RBCs, while in transplantation lower levels (100–200 pmol/8 × 10^8 RBCs) are proposed but have not been clearly associated with a better response to treatment (19).

In CD very few data have been published. The RBC 6TGN target levels remain controversial. A study by Cuffari et al. performed in adolescent CD with steroid dependent or steroid refractory CD and treated with 6-MP for more than 4 months showed a negative correlation between disease activity and RBC 6TGN levels (6). In a further work published as an abstract, a RBC 6TGN greater than 250 pmol/8 × 10^8 RBCs was proposed as a target level (11). Such a discriminant level was also suggested by an another team in a recently published work (10). The first study (6), has been criticized for its non-complete prospective design and the absence of clear definition of steroid dependent and refractory patients (20). In contrast, our study is fully prospective and was performed on well-defined subgroups of patients. In this condition our results do not confirm a significant correlation between RBC 6TGN and response to AZA/6-MP. Indeed, median RBC 6TGN levels were similar, around 160 pmol/8 × 10^8 RBCs, in patients in remission with AZA/6-MP alone and in patients still needing steroids to control their disease or patients with active disease despite AZA/6-MP. This absence of correlation as well as a relatively low RBC 6TGN level in responders to AZA/6-MP was also observed by Sandborn et al. in patients with steroid dependent disease (14). In this study a steady level of RBC 6TGN, around 160 pmol/8 × 10^8 RBCs, was already obtained after 2 weeks of treatment and remained stable for another 16 weeks. We also found that the level of RBC 6TGN remained stable even over 6 months, in patients in which the oral dose of AZA/6TGN was not changed. In a few patients, however, RBC 6TGN levels may vary significantly despite the absence of change in drug dosage. Interestingly, in 1 of our patients in remission under AZA and steroids RBC 6TGN levels decreased from 235 to 103 pmol/8 × 10^8 RBCs while AZA dosage remained unchanged. However, we can not exclude a lack of compliance therapy. Concomitantly, this patient experienced a relapse suggesting that in an individual patient a target level could be determined for efficacy. As shown earlier, however, this level seems particular to a given patient and can not be applied to the others. Obviously, in a larger population, as recently published, a statistically significant discriminant level may appear for the whole group (10). However, this must not occult the fact that many patients are responders to the treatment with lower RBC 6 TGN levels and that this 'discriminant level' is probably not very relevant from a clinical point of view, and has not to be considered as a universal target.

In our study, no significant correlation was found either between response to therapy and various haematological parameters. Particularly, as with Sandborn et al. (14), we could not confirm the association between response to AZA/6-MP and relative leukopenia. Analysing the various biological parameters together, the only significant correlation was between RBC 6TGN and MCV. This indicates that the MCV may to some extent reflect the accumulation of active metabolites in the erythrocyte (21).

No side effect of AZA/6-MP was observed in our patients over 6 months. This may be explained by the fact that all patients were already at inclusion under long-term therapy; the majority of patients with potential side effects having

already stopped the treatment prior to the study, which was thus not specifically designed for the investigation of side effects. As far as toxicity is concerned, Cuffari et al. proposed an association with high level of 6-MMP metabolite (6). The majority of our patients had no 6-MMP detected level and the only 3 patients in which it was detected had relatively low levels compared to Cuffari et al.'s patients. It must be noted that the sensitivity of our test was lower than the one of Lennard & Singleton (9). Therefore, some lower levels in the range 0.1–1 nmol/8 $\times 10^8$ RBCs may have been missed. However, according to Cuffari et al. (6), these levels do not seen to correlate with significant side effects. The relatively low concentration of RBC 6TGN and the absence of 6-MPP in patients non-responding to AZA (group 3) allowed us to increase the dosage of AZA with safety. Indeed, in these patients the increase of a mean of 0.5 mg/kg/day was not associated with any haematological complication over a 6-month period. Quite surprisingly, although, in 2 patients this increase in AZA dosage was followed by an increased RBC 6TGN concentration, in the 3rd patient a significant decrease was observed.

In conclusion, this work shows, contrary to preliminary studies, a broad overlap of RBC 6TGN levels as well as haematological parameters in patients in remission or not and responders or not to AZA/6-MP therapy. This suggests, beside a variability in the metabolism of these drugs, the existence of complex mechanisms of action. Nevertheless, beside the use of RBC 6TGN to confirm compliance to therapy, this dosage could be useful in non-responding patients, allowing, in the absence of leukopenia, to increase the dose of AZA/6-MP with safety. The specific utility of this dosage beside direct evaluation of methylation capacity (either genetic or biological) should be assessed in prospective studies.

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